3. Contents

Title

***In vitro* and *in silico* AHR assays for assessing the risk of heavy oil-derived polycyclic aromatic hydrocarbons in fish**

Aim

The objective of this study was thus to estimate the risk of heavy oil-derived PAHs in fish from Kesennuma Bay. We investigated the AHR transactivation potencies of six PAHs which were detected in the tissue of greenlings from Kesennuma Bay, using the *in vitro* rsAHR-driven reporter gene assay established in our previous study (Bak et al., 2013). The relative effective concentrations of these PAHs that induce 20% of the maximum benzo[α]pyrene (BaP) response ($REC_{20\text{-}BaP}$) or induce 20% of the maximum 2,3,7,8-TCDD response ($REC_{20\text{-}TCDD}$) and their respective REPs, which were compared to the potency of BaP or 2,3,7,8-TCDD, were obtained from the *in vitro* rsAHR responses to the PAHs. In addition, the binding potencies of the six PAHs to each rsAHR were estimated by *in silico* docking simulations. The relationship between the *in vitro* rsAHR transactivation and *in silico* binding potencies was examined and the applicability of an *in silico* approach for screening PAHs as rsAHR ligands was validated.

Procedure

1. *In vitro* rsAHR reporter gene assay

The *in vitro* reporter gene assays were carried out according to a method previously reported (Bak et al., 2013). The activation of each reporter vector was determined using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The luciferase activities in lysates were measured using a multimode microplate reader (BioTek Synergy2). The fold changes of the luciferase activity (firefly/Renilla) ratio in PAH-treated wells compared with those in control solvent wells were calculated. Data are presented as the mean ± standard deviation (SD) from six to eight technical replicates in two independent experiments.

2. Estimation of REPs and RECs

Dose–response curves of the PAHs for each rsAHR transactivation in the *in vitro* assay were plotted as relative units to the maximum response of BaP against logarithmically transformed doses. The 50% effective concentration (EC_{50}) values of PAHs for each rsAHR transactivation were obtained using GraphPad 5.0 (San Diego, CA). The $REC_{20\text{-}BaP}$ of each PAH was defined as the lowest observable effective concentration (LOEC). To compare the potencies of these PAHs with those of dioxin-like compounds (DLCs), $REC_{20\text{-}TCDD}$ values were also calculated for six PAHs and seven DLCs; 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF, 1,2,3,4,7,8-HxCDF, and PCB126. The raw data of each rsAHR transactivation in the *in vitro* assay from the seven DLCs were sourced from our previous paper (Bak et al., 2013).

REPs were estimated on the basis of a systematic framework which was previously proposed (Kim et al., 2011). BaP-relative potency 20, 50, and 80 (BaP-REP_{20}, -REP_{50}, and -REP_{80}) values were calculated as concentration ratios; the concentration that induces 20, 50, and 80% of the maximum

BaP response divided by the concentration that induces the corresponding response of each PAH, respectively. The BaP-REP of each PAH was expressed as the average of the BaP-REP_{20}, -REP_{50}, and -REP_{80} values.

3. *In silico* rsAHR homology modeling and docking simulations

The *in silico* homology modeling for the ligand binding domain (LBD) of rsAHR1 and rsAHR2 and the docking simulations of PAHs with the rsAHR LBD models were conducted using the Molecular Operating Environment, ver. 2015.10 (Chemical Computing Group Inc., Canada). The docking simulations were performed to estimate the potential binding energy of PAHs with each rsAHR LBD pocket using alpha sphere and excluded volume-based ligand-protein docking (ASEDock) provided by Ryoka Systems Inc., Japan. To construct homology models of rsAHR LBDs, we applied the crystal structure (PDB ID 3H7W.A) of the PAS-B domain sequence of human hypoxia-inducible factor 2α, which is a closely allied protein to the rsAHR. The results of the docking simulations of PAHs were shown as the binding potential energy, the U-dock value (kcal/mol). To establish the relationship between the *in silico* potential binding energy and the *in vitro* rsAHR transactivation potency, we additionally estimated the potential binding energies for the DLCs.

Result

1. rsAHR transactivation potencies of PAHs

To investigate the transactivation potencies of six PAHs for both rsAHR isoforms, an *in vitro* reporter gene assay system was applied. All the PAHs except 1-methylphenanthrene induced both rsAHR1- and rsAHR2-mediated responses in a dose-dependent manner. Exposure to 1-methylphenanthrene induced no response for either rsAHR isoform. The transactivation efficacies of dibenzothiophene and acenaphthene for rsAHR2 were higher than those for rsAHR1, whereas BaP, phenanthrene, and 2,3,5-trimethylnaphthalene gave similar efficacies for both rsAHRs. EC_{50} was calculated only for BaP because a full sigmoidal dose-response curve was obtained only for this chemical. The EC_{50} (0.016 nM) of BaP for rsAHR2 transactivation was 17.5-fold lower than that for rsAHR1 (0.28 nM).

The $REC_{20\text{-}BaP}$ values of phenanthrene, dibenzothiophene, acenaphthene, 2,3,5-trimethylnaphthalene, and BaP were 79, 38, 270, 88, and 0.052 nM for rsAHR1 and 53, 32, 60, 88, and 0.0049 nM for rsAHR2, respectively. The rsAHR2-derived $REC_{20\text{-}BaP}$ values of BaP and acenaphthene were lower than their respective rsAHR1-derived values, whereas phenanthrene, dibenzothiophene, and 2,3,5-trimethylnaphthalene had similar $REC_{20\text{-}BaP}$ values for both rsAHR1 and rsAHR2.

The BaP-REPs of phenanthrene, dibenzothiophene, acenaphthene, and 2,3,5-trimethylnaphthalene were calculated as an average of their respective BaP-REP_{20}, -REP_{50}, and -REP_{80}. Comparison of BaP-REPs among the examined PAHs showed that BaP had over 500-fold higher potencies than other PAHs.

In our previous study, the TCDD-REP values of seven DLCs were estimated using the same *in vitro* rsAHR1/2-derived reporter gene assay system (Bak et al., 2013). To compare the rsAHR transactivation potencies between PAHs and DLCs, the $REC_{20\text{-}TCDD}$ values of the PAHs were calculated. For rsAHR1, the $REC_{20\text{-}TCDD}$ (0.052 nM) and EC_{50} (0.28 nM) values of BaP were higher

than those of TCDD ($REC_{20\text{-}TCDD}$: 0.0064 nM and EC_{50}: 0.073 nM). In contrast, for rsAHR2, the $REC_{20\text{-}TCDD}$ (0.0049 nM) and EC_{50} (0.016 nM) values of BaP were lower than those of TCDD ($REC_{20\text{-}TCDD}$: 0.079 nM and EC_{50}: 0.52 nM).

2. *In silico* rsAHR-LBD homology modeling and PAH docking simulations

The docking simulations of each rsAHR with the six PAHs and seven DLCs were carried out using ASEDock. The lowest U-dock (kcal/mol) values were -23.4 kcal/mol of 2,3,7,8-TCDF for the rsAHR1-LBD model and -26.9 kcal/mol of 2,3,4,7,8-PeCDF for the rsAHR2-LBD model. The U-dock values of the six PAHs from the rsAHR1-LBD docking simulation ranged from -7.4 kal/mol for acenaphthene to -10.7 kcal/mol for BaP. For rsAHR2-LBD, the U-dock values ranged from -16.4 kal/mol for acenaphthene to -26.5 kcal/mol for BaP.

3. Relationships between *in vitro* and *in silico* measurements

To evaluate the results from the *in silico* docking simulations, we examined whether the rsAHR-specific $REC_{20\text{-}TCDD}$ values of the PAHs and DLCs obtained by the *in vitro* reporter gene assays could be predicted from the potential binding energies (U_dock values) derived from the *in silico* docking simulations. The U_dock values from the rsAHR1-LBD homology model showed a poor correlation with the rsAHR1-specific log-transformed $REC_{20\text{-}TCDD}$ values; Pearson R^2 =0.13 (p =0.24). In contrast, the U_dock values from the rsAHR2-LBD homology model showed a significant correlation with $REC_{20\text{-}TCDD}$ values; Pearson R^2 = 0.87 ($p < 0.0001$). This suggests that the rsAHR2-LBD model is more predictable for examining the *in vitro* transactivation potencies of PAHs than the rsAHR1-LBD model. The rsAHR2 model may thus be a useful tool for assessing the transactivation potencies of not only DLCs but also PAHs.

Publication/conference presentation

Bak, S. M., Nakata, H., Koh, D-H., Yoo, J., Iwata, H., Kim, E-Y. *In vitro* and *in silico* AHR assays for assessing the risk of heavy oil contaminated marine fish. SETAC AP 2018, Sep., 2018, Daegu, Korea, P42 O1-4.

Perspectives in future

Our *in vitro* rsAHR transactivation assay supported that PAHs were able to activate fish AHR in a chemical- and concentration-dependent manner. This study also suggests that AHR isoform- and species-specific transactivation potencies could be a critical toxicological issue for the further refinement of the risk assessment of PAHs in fish. This study provides an application of *in vitro* and *in silico* fish AHR ligand screening systems, in particular for PAHs. Further evaluation of the contribution of alkylated PAHs to AHR activation is necessary to better assess the risk of heavy oil contamination.